The GLOBAL CROP DIVERSITY TRUST
A Foundation for Food Security

Bioversity International

COGENT

CGIAR
RESEARCH PROGRAM ON
Forests, Trees and Agroforestry

Technical guidelines for the safe movement and duplication of Coconut (*Cocos nucifera* L.) germplasm using embryo culture transfer protocols

CA Cueto, VB Johnson, F Engelmann, A Kembu, JL Konan, M Kouassi Kan, RL Rivera, V Vidhanaarachchi, R Bourdeix and SF Weise

Bioversity International is a world leading research-for-development non-profit organization, working towards a world in which smallholder farming communities in developing countries are thriving and sustainable. Bioversity International's purpose is to investigate the use and conservation of agricultural biodiversity in order to achieve better nutrition, improve smallholders' livelihoods and enhance agricultural sustainability. Bioversity International works with a global range of partners to maximize impact, to develop capacity and to ensure that all stakeholders have an effective voice.

CGIAR is a global partnership that unites organizations engaged in research for a food secure future. CGIAR research is dedicated to reducing rural poverty increasing food security, improving human health and nutrition, and ensuring more sustainable management of natural resources. It is carried out by the 15 centres who are members of the CGIAR Consortium in close collaboration with hundreds of partner organizations, including national and regional research institutes, civil society organizations, academia, and the private sector. www.cgiar.org.

The International Coconut Genetic Resources Network (COGENT) is a network of coconut producing countries www.cogentnetwork.org/ promoting global collaboration to conserve and use coconut genetic resources. COGENT currently is made up of 39 countries members, responsible for more than 98% global coconut production.

Citation: Cueto CA, VB Johnson, R Bourdeix, F Engelmann, A Kembu, JL Konan, M Kouassi Kan, CM Oropeza Salín, RL Rivera, V Vidhanaarachchi, and SF Weise. 2012. Technical guidelines for the safe movement and duplication of coconut (*Cocos nucifera* L.) germplasm using embryo culture transfer protocols. COGENT; Bioversity International, Montpellier, France.

Cover photo credits: Pemba Red dwarf (PRD) in Cote d'Ivoire (left); Papua Red Dwarf Spicata (right), RBourdeix, COGENT, CIRAD Bios, Umr Cefe-CNRS.

Embryo cultured coconut seedlings (centre); VB Johnson, Bioversity International.

ISBN: 978-92-9043-924-0

Bioversity International
Headquarters
Via dei Tre Denari 472/a
00057 Maccarese
(Fiumicino) Rome, Italy

Bioversity International
France
Parc Scientifique Agropolis 2
34397 Montpellier Cedex 5
France

COGENT Secretariat
C/o Bioversity International
Parc Scientifique Agropolis 2
34397 Montpellier Cedex 5
France

Technical guidelines for the safe movement and duplication of Coconut (*Cocos nucifera* L.) germplasm using embryo culture transfer protocols

CA Cueto[1], VB Johnson[2], R Bourdeix[3], F Engelmann[4], A Kembu[5], JL Konan[6], M Kouassi Kan[7], CM Oropeza Salín[8] RL Rivera[9], V Vidhanaarachchi[10] and SF Weise[11]

[1] Philippines Coconut Authority, Albay Research Centre, Philippines

[2] Bioversity International, Montpellier, France

[3] COGENT, Montpellier, France; CIRAD Bios; Umr CEFE-CNRS, Montpellier, France

[4] IRD, Montpellier, France

[5] Cocoa and Coconut Institute of PNG, Madang, Papua New Guinea

[6] Coconut Research Programme, CNRA, Abidjan, Côte d'Ivoire

[7] Laboratoire de Biotechnologie, CNRA, Abidjan, Côte d'Ivoire

[8] Centro de Investigación Científica de Yucatán, Mexico

[9] Philippines Coconut Authority, Zamboanga Research Centre, Philippines

[10] Coconut Research Institute, Sri Lanka

[11] Bioversity International, Rome, Italy

Acknowledgements

Bioversity International would like to thank all those organizations and individuals who contributed to the development of these Technical guidelines for the safe movement and duplication of coconut (*Cocos nucifera* L.) germplasm using embryo culture transfer protocols. We particularly thank all the many organizations, institutions and individuals who have assisted us by unselfishly sharing their expertise and experiences: the Cocoa & Coconut Institute (CCI), Papua New Guinea; the Coconut Research Institute (CRI), Sri Lanka; l'*Institut de recherche pour le développement* (IRD) - UMR DIADE, France; the Philippines Coconut Authority (PCA), Philippines; la *Station de recherche Marc Delorme, Centre national de recherche agronomique* (CNRA), Côte d'Ivoire. We are especially grateful to the following individuals for their invaluable input: Dr Anitha Karun, Central Plantation Crops Research Institute (CPCRI), Kerala, India, Dr Haj Gunathilaka, Dr Chandrika Perera and Dr Lalith Perera and others from the CRI, Sri Lanka. We would also like to thank Dr Michel Dollet from CIRAD, Montpellier, France for the review work and photos in the section on coconut diseases, and Dr Carlos M. Oropeza Salín, *Centro de Investigación Científica de Yucatán*, A.C. (CICY), Mexico for his feedback in the external review. Finally we would like to acknowledge the Coconut Genetic Resources Network (COGENT) and the contributions of all those delegates who contributed to the three stakeholder meetings in PCA-ZRC, the Philippines (2008), in Abidjan, Côte d'Ivoire (2010) and in Lunuwila, Sri Lanka (2012).

We would also like to acknowledge the vital financial and in-kind contributions from The Global Crop Diversity Trust and Bioversity International.

A list of principal contributors is included at the end of this manual.

Contents

List of figures

Preface

The coconut palm (*Cocos nucifera* L.) is an important livelihood crop for millions across Southeast Asia, the Asia Pacific, Africa and Latin America. Fully developed and strategically used, coconuts could increase food production, improve nutrition, create employment opportunities, enhance equity and help conserve the environment. The future of global coconut production and livelihoods critically depends on the availability of genetic diversity and the sustainable use of this broad genetic base to breed improved varieties.

Harnessing and conserving agrobiodiversity are critical to sustainably boosting productivity and livelihoods, and addressing important challenges including those posed by climate change or pest and disease epidemics. Bioversity International continues to support the development of a progressive global strategy for conserving coconut germplasm. It aims to cost-effectively optimize conservation of as much representative diversity as possible. The safe and effective movement of coconut embryos can play a vital role in conserving and harnessing coconut germplasm. This includes ensuring optimal embryo (or plug) selection and transfer, according to strictly observed protocols.

Currently, coconut embryo transfer offers a feasible means of safely sharing coconut germplasm.

This 'Technical guidelines for the safe movement and duplication of coconut (*Cocos nucifera* L.) germplasm using embryo culture transfer protocols' is the main output of project work producing a protocol adapted from previously existing coconut embryo culture protocols (Frison et al, 1993; Batugal & Engelmann, 1998, 2002; Shivashankar et al., 1999; Rillo et al., 2002; Ikin & Batugal, 2004; Samosir et al., 2008). The project aimed to validate and apply the adapted protocol to provide users with an up-to-date, reliable method for effectively transferring coconut germplasm. Most importantly, the unique accessions housed in certain coconut genebanks need to be duplicated in other genebanks to minimise the risk of losing them. Embryo transfer offers an important facility for such duplication. This duplication work is now underway.

Bioversity International's overall coconut research findings are being reviewed in the light of upgrading the global coconut conservation strategy. In July 2012, the 16th Steering Committee meeting of the International Coconut Genetic Resources Network (COGENT) was held

in Kochi, Kerala, India. Agreements have been reached on finalizing the strategy, and designing further related research in terms of: conservation in national field collections; conservation in the multisite International Coconut Genebank (ICG); *in vitro* embryo culture, somatic embryogenesis and cryopreservation; *in situ* and on-farm conservation; promoting conservation through use, and 'Polymotu' - a concept employing geographic isolation to avoid costly controlled pollination.

Introduction

The coconut

The coconut palm (*Cocos nucifera* L.) is an important livelihood crop for many people in Southeast Asia, the Pacific Region, Africa and some countries in Latin America. It is one of the most economically important crops in the tropics, providing a good source of food, drink, shelter material, industrial raw materials and employment. Aside from the demand for coconut as copra, virgin coconut oil, coconut sugar, and coconut water, it is also a potential source of biofuels. Coconut is the second highest oil yielding plant after oil palm. Being an oil producing plant, it produces large amounts of carbon-sequestrating biomass, immobilizing CO_2 and thus, is an ideal crop system to help reduce the risk of climate change. Fully developed and strategically used, coconuts could increase food production, improve nutrition, create employment opportunities, enhance equity and conserve the environment.

The coconut faces several problems that can affect its production and competitiveness: low yield and yield security, and the unstable market for its traditional products, among others. Pests and diseases, repeated natural calamities, ageing of palms and genetic erosion further exacerbate coconut's vulnerability.

The availability and sustainable use of genetic diversity is critical to future of global coconut production and associated livelihoods partly though breeding improved varieties. Amongst coconut-producing countries there are 24 national and 5 international coconut genebanks. Efforts to manage coconut genetic resources effectively can only be carried out through international collaboration.

Bioversity International supports the ongoing development of a progressive global strategy for conserving coconut germplasm, aiming to effectively conserve as much representative diversity as possible, for the short, medium and long term.

Conservation of diversity

Conservation and management of coconut genetic resources include: targeted collecting, maintenance of field collections, effective characterization and identification, evaluation for important traits, information management, effective and safe exchange of germplasm and related information, and in some cases germplasm enhancement.

Since the early 1950s, numerous missions have been undertaken to collect and conserve coconut *ex situ* in genebanks. Created in 1992, the International Coconut Genetic Resources (COGENT) aims to strengthen international collaboration in conservation and use of coconut genetic resources; to promote improving coconut production on a sustainable basis, and to boost livelihoods and incomes of coconut stakeholders in developing countries. COGENT now comprises 39 country members.

To date, as shown in Table 1, 24 countries have provided germplasm data, including passport and field characterization descriptors, standardized pictures of germplasm (795 pictures) and molecular data (14 microsatellite markers observed on 462 populations and cultivars).

In 1999, the COGENT Steering Committee took the decision to release the coconut genetic resources database (CGRD) into the public domain, in order to make accessible and disseminate this useful information, and to create public awareness about coconut genetic resources. Version 6 of the database is now available on the COGENT website. According to the 2012 data, 24 national genebanks are conserving 725 unique populations with 1374 living accessions. 447 of these accessions, collected during the 1980s have not been rejuvenated.
(see http://www.cogentnetwork.org/index.php/cgrd-version-6-0-test-version)

The collection, conservation and utilization of coconut genetic resources and their global distribution are essential components of research activities underpinning the implementation of coconut improvement programmes.

Table 1. Documented data (as of April 2012) on national and international collections *(with international genebanks in orange).*

	Countries & Genebanks	# registered accessions	# active accessions	# unique active populations& cultivars	# Passport Data	# Characterization Data
1	Bangladesh Bari	40	16	16	699	932
2	Benin CRC Sémé Podji	4	0	0	116	20
3	Brazil EMBRAPA	29	23	23	1 286	1665
4	China Wenchang Coconut Research Inst.	17	17	3	366	312
5	Côte d'Ivoire CNRA Marc Delorme R. S.	149	124	61	5 782	10 163
6	Fiji Taveuni Coconut Centre	11	10	10	413	536
7	Ghana OPRI	16	15	13	206	71
8	India CPCRI	301	299	170	9 227	16 122
9	Indonesian Palm Research Institute	203	84	62	5 747	6 631
10	Jamaica Coconut Industry Board	60	47	28	923	522
11	Malaysia Dept. of Agric. Sabah	45	37	19	968	924
12	Malaysia MARDI Hilir Perak	44	44	44	1 213	2 621
13	Mexico CICY Yucatan	20	0	0	568	800
14	Pakistan	32	0	0	192	1148
15	Papua New Guinea Stewart Res. Centre	57	50	49	937	759
16	Philippine Coconut Authority	224	224	130	6 444	8 259
17	Solomon Islands Yandina Res. Centre	21	20	20	398	147
18	Sri Lanka Coconut Research Institute	157	154	127	6 322	10 950
19	Tanzania Nat. Coconut Dev. Programme	72	65	57	2 420	963
20	Thailand Chumphon Hort. Research Centre	52	51	49	1 480	760
21	Tonga Ministry of Agriculture	7	0	0	45	274
22	Vanuatu Saraoutou Research Centre	79	57	44	2 494	1 768
23	Vietnam Dong Go Experimental Centre	31	31	31	1 221	1 093
24	Western Samoa	9	6	6	84	36
	Total	**1 680**	**1 374**	**962**	**49 551**	**67 476**

Limitations of field genebanks

For coconuts, field genebanks are the most common means for *ex situ* germplasm conservation. However, the use of coconut field genebanks presents a number of constraints. In classical coconut genebanks, coconut cultivars are conserved as accessions, generally planted close together in the same fields. Each accession usually counts 75 to 100 coconut palms from the same cultivar. They require a large field area to accommodate the large numbers of accessions. Maintaining this amount of material and land area is therefore highly labour- and time-intensive and very costly to manage. Furthermore, because of the need to access exactly the diversity as specified, extreme care and precision is needed in labelling accessions and managing the field genebank. For reproducing these accessions, the technique of controlled pollination with bagging of the inflorescence is used (Konan et al., 2008). For coconuts, this technique is very costly. It requires a well-equipped laboratory for pollen processing, well-trained technicians able to climb the palms and a huge amount of manpower. Not all the genebanks can afford this. The average lifespan of such accessions is only 25 to 30 years. After this period, most non-dwarf coconut varieties reach 15 m high or more. At this stage, it becomes difficult to make the requested controlled pollinations. It is therefore necessary to rejuvenate the accessions before the inflorescences become inaccessible. In the Côte d'Ivoire African genebank, workers use costly triple ladders that can reach a height of only 14m. In some other places, like India or Indonesia, palms are climbed mainly manually, which is risky. Rejuvenation programmes require climbing roughly 75 palms each about 15-20 times. Basically, for rejuvenating an accession, the controlled pollinations are implemented over a six-month period; the mature seednuts are harvested one year later, also over a six-month period; then the old accession is removed from the field and replaced by a new one. Production of the 200 seednuts required for the duplication of an accession will demand one and half year's preparation; and it will cost more than 2000 USD. Only scientists with healthy research budgets can afford to order varieties from classical coconut genebanks (Bourdeix et al., 2012). Most farmers cannot manage to pay for this.

The living accessions maintained in field genebanks are also subject to several biotic and abiotic factors that threaten their survival. These factors most commonly include: pests and diseases; sub-optimal temperatures,

water supply and soil nutrients; other climate effects, political crises, and land conversion.

Germplasm exchange between field genebanks is often hampered by several constraints like large size of the nut, lack of dormancy and phytosanitary regulation. These limitations could be overcome with coconut embryo culture technique which was developed to rescue the non-germinating Makapuno variety (de Guzman and del Rosario 1946). The technique has been very useful in germplasm collecting, exchange and conservation a well as in *in vitro* screening for some biotic and abiotic factors (i.e. disease, screening for disease resistance and drought tolerance). It has been used to transfer coconut germplasm with varying levels of success within and between countries (Batugal and Engelmann, 1998; Engelmann et al., 2011).

There is an urgent need to conserve coconut germplasm, especially the unique accessions. However, the lack of a robust method for transferring and exchanging coconut germplasm has posed a limitation in advancing the conservation of coconut genetic resources.

Towards safe movement of coconut germplasm

A rationale for the guidelines

A critical element of the global strategy to conserve coconut genetic resources is establishing a robust protocol for the safe movement and duplication of coconut germplasm.

Because of its large, recalcitrant seed that exhibits no dormancy, coconut diversity is conserved in field genebanks, which until recently has been the only practical method for coconut *ex situ* conservation. They however require a large area and substantial resources to maintain, and are subject to many risks such as exposure to pests, diseases, abiotic stresses and natural and man-made calamities. Many countries also lack the capacity and financial resources to maintain their collections.

As mentioned previously, collections that represent coconut diversity are located in 24 national and 5 regional genebanks, and in the multi-site International Coconut Genebank. The regional field genebanks are established, maintained and managed by national programmes with

guidance from the International Coconut Genetic Resources Network (COGENT). This was founded by Bioversity in 1992, as a global network of coconut producing countries, aimed at improving the production and use of coconut and the conservation of its diversity.

As another potential means of ensuring long-term conservation, accessions can now also be cryopreserved, that is frozen to the temperature of liquid nitrogen (-196°C). Cryopreservation arrests both the growth of plant cells and all processes of biological deterioration, so that the material can be preserved for extended periods and resuscitated into fully viable plants. The one-off cost of cryopreserving accessions is expected to pay off against the recurrent costs of *in vitro* or in field maintenance over a number of years. In December 2012 Bioversity commenced new cryopreservation work in Korea.

The guidelines presented in this booklet represent a summary of the findings arising from grant work entitled: "Validation of a coconut embryo culture protocol for the international exchange of germplasm". The work has been funded by the Global Crop Diversity Trust, and coordinated by Bioversity International, in partnership with the Cocoa & Coconut Institute (CCI), Papua New Guinea; the Coconut Research Institute of Sri Lanka (CRI), Sri Lanka; the *Institut de recherche pour le développement* (IRD) - UMR DIADE, France; the Philippine Coconut Authority, Philippines, and the *Station de recherche Marc Delorme, Centre national de recherche agronomique* (CNRA), Côte d'Ivoire.

The project was implemented via four (4) laboratories from Côte d'Ivoire (CNRACI); Papua New Guinea (Cocoa and Coconut Institute - CCIPNG); the Philippines (Philippine Coconut Authority - Zamboanga Research Centre - PCAZRC), and Sri Lanka (Coconut Research Institute - CRISL). Each has an important national coconut genebank collection with existing tissue culture laboratories. These countries participated in the multi-country experiment to validate and develop a robust method for the international exchange of coconut germplasm. CNRACI, which manages the International Coconut Genebank for Africa and the Indian Ocean (ICG-AIO), was able to supply embryos of Malayan Yellow Dwarf (MYD) and West African Tall (WAT) for the protocol validation phase and of the 11 unique accessions for regeneration in PCAZRC and CRISL. Moreover, it was able to supply the embryos of the dwarf accessions identified by the partners.

In the medium term coconut embryo transfer seems to offer the most reliable and cost effective means of safely sharing coconut germplasm. The overall aim of the grant work has been to optimize, validate and apply a standard embryo culture protocol for wide application under a variety of genotypes and conditions. The specific objectives were to:

1. test and optimize embryo culture techniques through the transfer of embryos from the ICG-AIO in Côte d'Ivoire to the Philippines, Papua New Guinea (PNG) and Sri Lanka;
2. assess the feasibility of shipping embryos as compared to hand-carrying embryos;
3. produce technical guidelines for the successful exchange of coconut germplasm as embryos;
4. duplicate 11 threatened accessions in the ICG-AIO in other genebanks using the validated protocol to transfer embryos.

An international inception workshop was held in Abidjan, Côte d'Ivoire, in which participants agreed on a flexible coconut embryo culture protocol. This helped ensure greater consistency and effectiveness of subsequent embryo culture work. Protocol validation and application work has been completed. The final project workshop, held in Lunuwila, Sri Lanka in February 2012, synthesised the findings of the grant work and furnished recommendations incorporated into this edition of the protocol.

The project findings indicate that coconut embryo culture technology could provide an effective means for movement and exchange of coconut germplasm. However following embryo extraction, culture and transfer, the efficiency of the embryo culture protocol tested for germplasm exchange was generally low. High contamination and poor germination of the embryos contributed to the low efficiency. Contamination via culture medium has constrained recovery percentages of the coconut embryos. Annex A presents a data summary for the coconut embryo transfer work. Varying and low levels of embryo contamination (10-98%) and germination (0-90%), and subsequent seedling establishment indicate the need for protocol adherence. *In vitro* manipulation enhanced success rates.

The success of the technology is dependent on the situation in the field, laboratory, screenhouse and the technical expertise in the originating and recipient laboratories. Moreover, the success is also dependent on the political situation in the countries involved in the exchange programme. Survival and establishment of embryos and embryo cultured seedlings have confirmed the effects of genotype, age of nut, disinfection and storage procedures, access to

and level of technical expertise and effective laboratory, screenhouse and tissue culture facilities. The unstable political situation in Côte d'Ivoire has also greatly constrained the implementation of the project.

Whilst the project has not succeeded in duplicating the 11 targeted unique accessions, at least partial duplication of some accessions has been achieved.

Project work has substantially refined the previously existing embryo transfer protocol. However, to ensure the effective implementation of the protocol, both providers and recipients of the embryos should be equipped with the appropriate skills, and the required equipment should be available, in good working order which fully respects the revised protocol. The movement of embryo cultured seedlings should also be formally assessed to determine the feasibility of transporting seedlings instead of embryos especially to genebanks without tissue culture facilities.

As a result of this work, as well as safeguarding the intrinsic biodiversity value of such germplasm conservation, we hope that ultimately those depending on coconut production are likely to enjoy greater food and livelihoods' security.

The technical guidelines

This manual aims to provide working technical guidelines for the transfer and exchange of coconuts *in vitro* and the embryo culture protocol for growth and development of coconut seedlings including those intended for safety duplications of coconut germplasm.

The guidelines are intended to provide the best possible coconut *in vitro* transfer protocol and phytosanitary information to institutions involved in coconut germplasm exchange for research purposes.

Coconut breeders and tissue culture experts contributed to the formulation of the technical guidelines in their personal capacity and do not represent or commit the organizations for which they work.

Bioversity International and the contributing experts cannot be held responsible for any problems resulting from the use of the information contained in the technical guidelines. These reflect the consensus and knowledge of the specialists who have contributed to this revision but the information provided needs to be regularly updated. The experts who contributed to the production of these technical guidelines are listed in this publication.

General guidelines and recommendations for coconut *in vitro* transfer

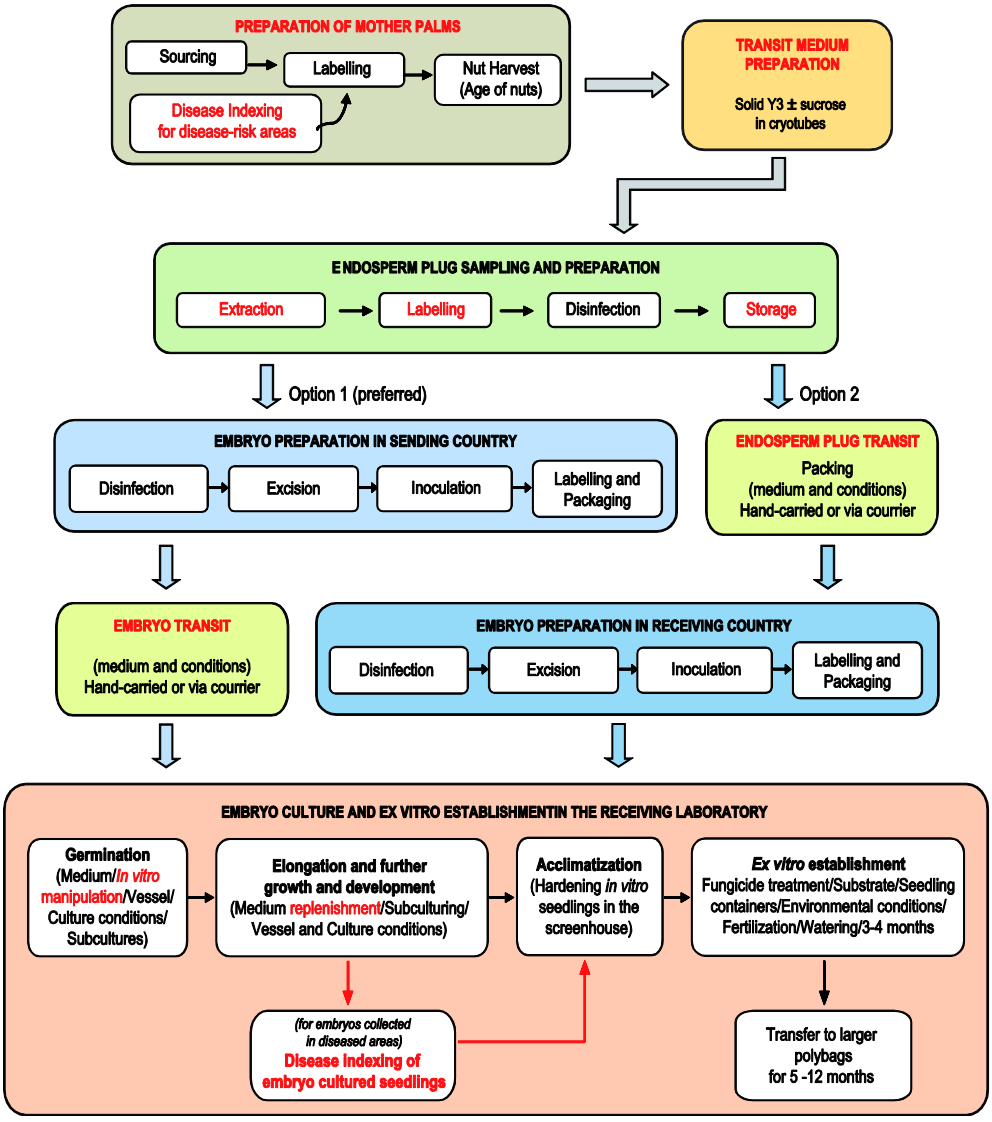


Figure 1. Stages in coconut embryo transfer (CA Cueto, VB Johnson & R Bourdeix).
(Red text indicates additions to earlier versions of the protocol).

Figure 1 illustrates the key stages in coconut embryo transfer, from selection and preparation of the mother palms in the donor country to the *ex vitro* seedling establishment in the recipient country. The protocol is described in the subsequent sections.

The coconut embryo culture transfer protocol

Arrangements in donor and recipient countries

1. During the exchange of germplasm of any accession, a control sample (one rack of 24 embryos) should be established in the country of origin as *in vitro* materials. This provides for evaluating germination and contamination levels, in case there is a problem with the samples dispatched to the receiving genebank. The sample should be kept for 2-4 months after collection. The acceptable levels of germination and contamination are to be agreed between country of origin and collecting country.
2. In the case of hand-carried embryos, collecting in the country of origin should be performed by staff of the recipient country.
3. Detailed planning and communication between collecting and recipient countries is essential.
4. The deployment of resources (especially time) for the whole process of embryo extraction and transfer needs to be clearly articulated.
5. Phytosanitary related documents of the importing and receiving countries must be properly drafted, filed and available prior to shipment of the embryo cultured materials (embryos or seedlings)(see pages 55-59).
6. A fully operational and well-maintained tissue culture facility with the basic tissue culture equipment and supplies, as well as technical staff should be available in the genebank to ensure smooth flow of collecting and tissue culture activities.
7. Cleanliness of the laboratory at the highest standards possible must be maintained.
8. Contamination level must be maintained at the lowest level possible (maximum 10%). Appropriate laboratory procedures to maintain cleanliness and orderliness (e.g. regular fumigation of culture rooms) should be established.

Selection and preparation of mother palms

1. The collecting team should include one coconut breeder (for selection of palms to be collected and collection of passport data) and one tissue culture specialist.
2. Identify healthy looking and high yielding mother palms.
3. Ideally, embryos are to be sourced from disease-free areas. When harvesting and processing of nuts in disease-risk areas, assay the mother palms for absence of the pathogen. There are molecular diagnostic tests available to detect the presence of the pathogen: i.e. molecular assay for the coconut Cadang-cadang viroid (CCCVd) (Rodriguez, 2011) and phytoplasma belonging to the 16SrX1 *Candidatus Phytoplasma oryzae* group and causing Weligama coconut wilt disease (Perera et al., 2012).
9. Results of disease diagnostic assays of mother palms should be provided to the receiving country.
10. The pollination date of the mother palms must be noted for the exact time of harvest.

Harvesting and processing of nuts

1. Harvest nuts of Tall coconut accessions from controlled pollinated Tall and of Dwarf coconut accessions for the self-pollinating Dwarfs.
2. Eleven month-old nuts are ideal for embryo culture; 10 months for the Dwarf and 11 months for the Tall. Usually this is when at least one nut in the bunch turns from the fresh to the dry colour (Frison et al., 1993) Over-mature nuts have germinated *in situ* whereas immature nuts will not readily germinate *in vitro* (Figures 2 and 3)
3. For safety duplication of palms in coconut genebanks, at least 90 palms for Tall and 45 palms for Dwarf coconut should be field-planted. Targeting a 30% success for embryo culture requires inoculating 300 embryos from Tall and 150 embryos from Dwarf coconuts. Therefore, there is a need to harvest more than 300 Tall and 150 Dwarf embryos to give allowance for nuts that have germinated, spoiled meat, damaged embryos and endosperm plugs without the embryos.

Figure 2. Over-mature, germinated coconut (CA Cueto, PCA-ARC).

Figure 3. Recognising 10-11 month old nuts (RL Rivera, PCA-ZRC).

Labelling of palms, nuts, embryos and seedlings

1. The traceability of the embryos is of utmost importance. The labelling of the embryos must be retained until field planting of resulting plantlets. Data to be included in the label are the variety, and the number of the mother palm for open pollinated and the control pollination number for the hand-pollinated nuts.
2. It is necessary to precisely record the variety/accessions of each of the nuts. Label the bunches of nuts on a per palm basis. Label the nuts according to the number of the mother palm if they are harvested from open pollinated palms. On the other hand, label the nuts based on the Controlled pollination number if they are harvested from controlled-pollinated palms (Figure 4).
3. To maintain the traceability of the palms, the labels of the embryos and seedlings in the laboratory and in the screenhouse and nursery should be retained until and including when they have been field-planted in their respective genebanks.
4. In a clean working environment, de-husk the seednuts (Figure 5).

Figure 4. Nut labelling
(RL Rivera , PCA-ZRC).

Figure 5. De-husking
(RL Rivera, PCA-ZRC).

5. Split the de-husked nuts into halves by striking the longitudinal vein that runs around it with a hard metal rod or sharp 'bolo', a machete-like cutting tool (Figure 6).
6. Discard any nuts with germinated embryos and/or rotting solid endosperm or meat. Spoiled meat could contribute to the contamination of the embryo.

Figure 6. Nut splitting (RL Rivera, PCA-ZRC).

Processing of endosperm plugs

Extraction of endosperm plugs

1. The number of endosperm plugs and embryos to be cultured depends on the accession and status of the laboratory and expertise available in the collecting site.
2. Batch-wise collecting of endosperm plugs is advised to ensure smooth and good quality of processing of the embryos.
3. During aseptic technique, a maximum of 150 embryos for each person per day is feasible due to the labour intensiveness of embryo traceability labelling.

4. Under the fertile eye of the coconut, extract the endosperm plug embedding the embryos with care, using a sharp[12] serrated cork borer (Figures 7 and 8). Avoid exposing and damaging the embryo.

Figure 7. endosperm plug extraction
(RL Rivera, PCA-ZRC)

Figure 8. Serrated cork-borer
(VB Johnson, Bioversity International)

5. Expel the endosperm plug from the cork borer using a blunt plunger which could be made of wood. The use of hammer to push out plugs should be avoided to minimize embryo damage.
6. Discard plugs with germinated, exposed and/or damaged embryos. The testa covering the endosperm plugs should be intact, brown in colour but not too dark (too mature) or too light (immature).
7. Collect the endosperm plugs in properly labelled, clean/sterile containers. Avoid mixing endosperm plugs harvested from the different palms and accessions.
8. Work with one accession at a time.

Disinfection of endosperm plugs

1. Protect hands from irritating effects of commercial bleach with gloves.
2. Wash endosperm plugs with detergent. Rinse thoroughly with tap water to remove the detergent.

[12] Ensure sufficient cork borers and a sharpener are available (C Oropeza, pers. comm. 2012)

3. Wash endosperm plugs for a few seconds with 70% ethyl alcohol to remove excess oil (if any).
4. Disinfect endosperm plugs with 100% commercial bleach (5.25% a.i. sodium hypochlorite) for 20 minutes with occasional stirring (Figure 9).
5. Rinse the bleached-treated endosperm plugs 3-5 times with sterile water to remove the bleach.

Figure 9. Coconut endosperm-plug disinfection (RL Rivera, PCA-ZRC).

Storage of endosperm plugs

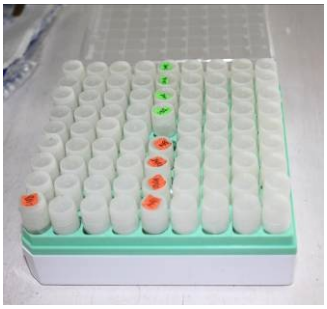
1. In cases where the endosperm plugs could not be processed immediately for embryo culture, store the endosperm plugs in sterile polypropylene (autoclavable) bags.
2. Seal and label the bags accordingly: variety/accession, control pollination number or number of mother palms, whichever is applicable and date of collecting/processing (Figure 10).
3. For transit from collecting/processing site to the laboratory within the country of origin, keep the sealed polybags with endosperm plugs in Styrofoam boxes with ice.

4. In the laboratory if embryo excision could not proceed yet, keep the endosperm plugs overnight in a refrigerator at 10-15°C; process them for embryo excision within 24 hours. In exceptional cases, the endosperm plugs can be stored for up to 2 days at 10-15°C.
   With regard to embryo refrigeration, storage of the embryos in the refrigerator below 15°C inhibits their germination. It is recommended that a maximum-minimum thermometer is inserted in cool boxes and refrigerators where embryos are stored to monitor any temperature extremes.

Figure 10. Packing and labelling endosperm plugs (RL Rivera, PCA-ZRC).

Preparation of materials for transit

Transit vials



1. Prepare the cryotubes for sterilization: 2 ml, polypropylene, screw cap cryotubes are ideal to store the embryos during transit.
2. The 81-hole cryobox to contain the cryotubes are preferred to store the cryotubes during transit. If available, an autoclavable cryobox is preferred (Figure 11).

Figure 11. Cryotubes in box for transit (PCA-ZRC).

Transit medium

1. It is recommended that embryos are transferred in at least 5-day old solid Eeuwens' (Y3) medium (Annex B, Table B1) with or without 0.75% sucrose and 0.6% agar. Evidence from Pech y Aké et al. (2004) indicates 'that percent germination [is] greater when embryos [are] cultured in solid medium'.
2. Take out the Y3 stock solutions from the refrigerator.
3. Weigh the sucrose and gelling agent.
4. Measure out the following from the stock solutions (Table 2):

Table 2. Concentration of stock solutions and volume of stock solution to prepare for 1x of 1 L Y3 medium.

Stock solution	Volume for 1 L of Y3 medium (ml)
Macronutrients (10x)	100
Micronutrients (100x)	10
Vitamins (100x)	10
FeEDTA (100x)	10
Myo-inostiol (100x)	10

5. Add the sugar (if required), stir until dissolved.
6. Adjust volume as required.
7. Adjust pH to 5.7.
8. Add gelling agent. Dissolve gelling agent by heating. Stir occasionally.
9. Mix all components very well.
10. Dispense the medium in flasks or bottle. Wipe the mouth of the culture vessel to remove adhering medium. Cover the flask/bottle.
11. Sterilize the following at 121°C at 15 psi for 20 minutes:
    - Y3 medium in covered flasks or bottles,
    - Clean, empty cryotubes with loosened screw caps in cryobox (if cryobox is autoclavable) or in autoclavable plastic bags,
    - Micropipette tips or 10-ml glass pipettes with cotton plugs (if no micropipettor available) individually wrapped or in canister.
12. Cool down the medium and cryotubes to around 65°C.

13. Inside the laminar flow cabinet, under aseptic condition, dispense 1 ml Y3 medium to individual cryotube using sterile glass pipettes or clean micropipettor with sterile micropipette tips.
14. Wait for the medium to solidify. Cool before use. Preferably use immediately, although it is useful to wait for about three days to check on the contamination of the media.
15. If cryotubes with medium will be not used immediately:
    - Tighten the screw-caps of the cryotubes,
    - Protect the cryotubes inside the cryobox by putting them inside sterile plastic bags,
    - Store in clean, dry place or inside the refrigerator for not more than one week.

Additional preparations:

1. In addition to the items sterilized with the medium, sterilize the following before processing the embryos:
    - Flasks, beakers or bottles to contain the endosperm plugs inside the laminar flow,
    - Petri dishes individually wrapped or in canister,
    - Empty glass containers for the embryos during disinfection and after excision,
    - Empty graduated cylinders covered with aluminium foil or keep in autoclavable plastic bags,
    - Distilled water for rinsing,
    - Sets of scalpel blades and pairs of forceps,
    - Empty big bottles to contain waste during disinfection and rinsing of endosperm plugs and embryos.
2. Cool the items before use.
3. Inside the laboratory, wear appropriate laboratory clothing: gown, mask and hair net.
4. Prepare the laminar flow cabinets:
    - Spray/wipe it with 70% ethyl alcohol,
    - Air-dry,
    - Turn on the UV lamp for at least 30 minutes before use,
    - Turn off UV lamp,
    - Turn on the fan and leave on for at least 20 minutes before using,
    - Turn on the glass bead sterilizer (if any) to attain 250°C,
    - Turn on the lights.

5. Spray hands and everything that will be brought inside the cabinet with 70% ethyl alcohol. If using an alcohol lamp, be careful not to light the lamp with hands and other materials inside the cabinet, still wet with ethyl alcohol to avoid burning.
6. After using the laminar flow cabinet:
   - Turn off the glass bead sterilizer,
   - Clean the laminar flow by spraying the inside of the cabinet with 70% ethyl alcohol,
   - Turn off the lights,
   - Turn off the fan when the alcohol has dried up,
   - Close the laminar flow cabinet.

Preparation of embryos

Disinfection and excision of the embryos

(see Figure 12)

1. Take note of the labels on each of the plastic bags and its contents. Spray the plastic bags containing the endosperm plugs thoroughly with 70% ethyl alcohol.
2. Inside the laminar flow (under aseptic conditions), take out the endosperm plugs and transfer to sterile container(s); number of containers will depend on the number of accessions and endosperm plugs.
3. Re-disinfect with 10% commercial bleach to reduce contamination (Carlos et al pers. comm.).
4. Using a scalpel blade and a pair of forceps, excise the embryos.
5. Collect the excised embryos in sterile container
6. Disinfect the excised embryos with 10% commercial bleach (5.25% a.i. sodium hypochlorite) for 5-7 min.
7. Rinse the disinfected embryos three to five times with sterile water to remove the bleach. Transfer the embryos to a sterile container with a small amount of water until ready for inoculation.
8. Be sure to maintain the labels properly. Avoid mixing the embryos.

Inoculation of the embryos

(see Figure 12)

1. Inoculate the embryos singly onto Y3 solid medium contained in 2 ml cryotube. Evidence from Oropeza et al. (2004) indicates that 'percent germination [is] greater particularly when embryos [are] placed with their micropyle end facing upwards in relation to the vial orientation, independently of orientation in relation to gravity. This [occurs] because solid medium [allows] embryos to be positioned with their micropyle end exposed to the ambient atmosphere of the vial'.
2. Label each cryotube accordingly. Use Sharpie or Pilot permanent markers which will withstand the alcohol during spraying of the labelled cryotubes. Perform a test to verify that the ink is indeed resistant to alcohol spray.
3. Seal the cryotubes with the embryos individually with clingwrap or parafilm
4. Arrange cryotubes in cryoboxes according to the label (see Figure 11). Always include a hard copy of the details of the embryos in the package to be shipped. For embryos coming from different accessions and contained in one cryobox, include a diagram on how the cultures are arranged in the cryobox.
5. Via email or courier, provide the receiving laboratory a duplicate copy of the details of the shipped embryo cultures.

Options for transporting coconut embryos and embryo cultured seedlings *in vitro*

1. Prior to shipment, the inoculated cryovials with embryos and polypropylene bags with the embryo cultured seedlings are kept for three days in the laboratory of origin to check on the contamination of the cultures. Level and type(s) of contamination must be determined; contamination level should not exceed 10%, otherwise, the whole batch will not be transported and sent to the receiving laboratory.
2. Coordinate with the receiving laboratory on the number of embryos to be sent and date of dispatch for the necessary media preparation. Moreover, inform the receiving laboratory of the details, i.e. name of courier and airway bill number.

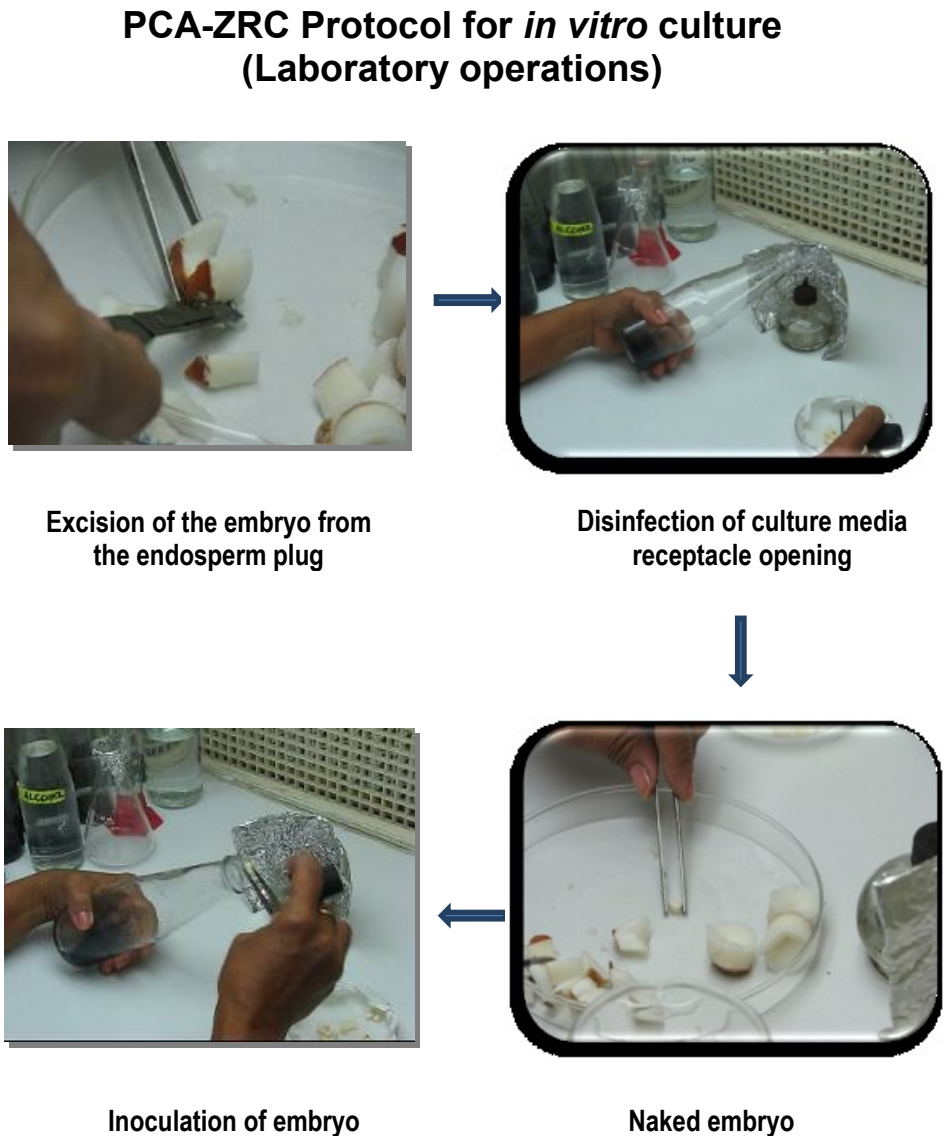


Figure 12. Embryo excision and inoculation (PCA-ZRC).

3. Embryos should be placed in boxes together with the necessary phytosanitary certification (see pages 55-59) and details of the embryos in cryotubes and/or embryo cultured seedlings in sterile plastic bag with Y3 solid medium to be either hand-carried or sent via courier to the receiving laboratories.

Embryo culture

1. Embryos in the culture medium are prepared as described above. Embryos are then cultured in the receiving laboratory according to their respective embryo culture protocol. (see Engelmann et al., 2002).
2. Only non-contaminated, clean embryos and seedlings in cultures are maintained and sub-cultured in the embryo culture medium.

Embryo cultured seedlings

1. In the absence of tissue culture facilities and /or technical expertise, the embryos in culture are sent to a laboratory wherein the embryos will be established until such time that they are ready for *ex vitro* establishment and for shipment/transfer to the requesting laboratories. Only embryo cultured seedlings with fully developed shoots, at least one fully expanded leaf and with primary and secondary roots and are ready for *ex vitro* establishment will be transported (Figures 13 and 14).
2. The availability of a screenhouse facility and technical expertise and support to establish the seedlings *ex vitro* should be ensured for the transfer of the embryo cultured seedling while still in the culture medium.
3. Label the embryo cultured seedlings in polypropylene plastic bags with permanent markers which could withstand the alcohol during spraying to ensure traceability of the seedlings.
4. The seedlings while still in culture medium will be acclimatized in the nursery for one week in the receiving country before transplanting them to the soil. This will give enough time for checking for the contamination acquired by the cultures during transit and for the seedlings to recover from stress during transit.
5. If the receiving laboratory does not have its own *ex vitro* establishment protocol, the protocol described hereafter should be used.

6. Arriving cultures containing embryos and/or embryo cultured seedlings should be checked for contamination. Contaminated cultures will be discarded and autoclaved to ensure death of any incoming microorganisms.

Note: If embryos and embryo cultured seedlings are coming from disease-risk areas i.e. areas reported with coconut Cadang-cadang viroid and Phytomonas-infected coconut palms, the FAO/IBPGR recommendation would be adopted (FAO/IBPGR 1994).

---

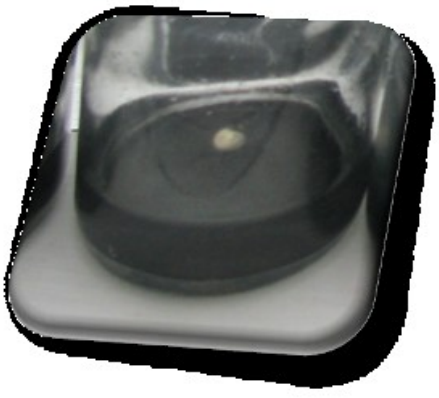
7. When plantlets are well-developed (at least one fully expanded leaf and one or more principal roots>3cm), a sample should be taken of each for indexing, using the following procedure:
   - Cut approximately 0.5 g (equivalent to 10 cm) from the distal part of the youngest expanded leaf, wipe free of moisture or culture medium and seal in a plastic bag.
   - Keep samples cool (but do not freeze) and immediately consign by courier or air freight to the indexing laboratory, enclosing an import permit issued by the receiving country.
   - Notify the testing laboratory by telex or fax when the samples are dispatched.
8. Materials should be released only when the indexing procedures confirm freedom from viroids and phytoplasma where appropriate.

Figure 14. Four month-old embryo cultured seedling ready for *ex vitro* establishment
(C Cueto, PCA-ARC)

PCA-ZRC Protocol for *in vitro* culture (Shelves/racks)

Embryos were singly inoculated onto "ketsup" bottle containing Y3 liquid media

It is lighted with 40 watts fluorescent tube with approximately 4000-5000 lux just above (6 inches) the bottles at 9 h photoperiod (15 h dark and 9 h light)



Developing embryo

Germinating embryo

Figure 13. Embryo culture and germination (PCA-ZRC.)

Mode of transfer

The embryos and the embryo-cultured seedlings can be dispatched from country of origin either by hand-carrying or via courier. Table 3 summarises comparison of hand-carrying versus couriering.

Table 3. Comparing transport by courier and hand-carrying.

Parameters	Hand-carrying	Via courier
Cost	Entail additional expense of travelling specialist to collect embryos	Entail cost of freight
Duration of transit	Depends on the distance of the originating laboratory to the receiving laboratory. Normally, a maximum of four days.	Depends on the courier service provider; could last up to two weeks. Can take sometimes for the package to be released due to custom-related issues
Handling during transit	Assurance of proper handling and storage conditions of the package	No assurance of the storage conditions provided by the courier service.

When hand-carrying, the person transporting the embryos or plugs should keep the package as hand-luggage. For this to happen it is very important to ensure that prior arrangements are made with the airline concerned. Important issues include: any necessary permits, observing baggage weight limits, and choosing the shortest and safest routing (Oropeza, pers. comm. 2012).

The embryo culture protocol (in the recipient country)

1. The Eeuwens' (1976) or the "modified" medium (Rillo et al., 2002) (see Annex B, Table B2) is used as the basal medium. The protocol may vary slightly depending on the receiving tissue culture laboratory.
2. Upon arrival in the laboratory, un-pack the boxes carefully ensuring that the cryovials are not mixed up and the labels of each tube with the embryo is intact.
3. Examine the cultures for presence of the contaminants. Discard and autoclave contaminated cultures to kill the microorganisms. Autoclaving will prevent introduction of new microorganism(s) that could have been acquired from host country or during transit.

Inoculation and germination of the embryos

1. Spray the cryotubes containing contaminant-free cultures thoroughly with 70% ethyl alcohol (having ensured labels will not be compromised[13]) before bringing them inside the laminar flow cabinet.
2. Under aseptic conditions, use sterile forceps to transfer the embryos singly to the germination liquid Y3 or "hybrid 2002" medium (see Annex B, Table B2). Retain the labels of the embryos and maintain until further development of cultures and establishment in the screenhouse, nursery and field.
3. Allow three months for the embryos to germinate (Figure 15).
4. After 2-3 months, remove the shoot cap covering the shoot tip of any ungerminated and/or slow germinating embryos (Figure 16).
5. This type of in vitro manipulation of the embryos exposes the shoot tip, thereby, promoting their germination and allowing their subsequent development. Subculture the decapitated, slow germinating embryos in Y3 or "hybrid 2002" solid medium supplemented with 0.16mg/L filter-sterilized gibberellic acid (GA3) or 10mg/L benzyl amino purine (BAP). Incubate the GA3-treated embryos for 1 month in the dark and the BAP-treated embryos under 9 h and 16 h photoperiod (25µM/m2/s) for PCA-ARC and CRISL, respectively, for one month.

[13] e.g. using 'Sharpie' markers.

Figure 15. Embryos three months after germination
(CA Cueto, PCA-ARC).

Figure 16. Removed shoot cap
(CA Cueto, PCA-ARC)

Notes:

Pech y Aké et al. (2007) report that using Gibberellic acid (GA3) can benefit coconut embryos in culture, favouring germination and conversion for plants grown in a semi-solid medium.

For GA3-supplemented media, the GA3 should be filter-sterilized using Millipore filtration:

- Sterilize the filtration set-up, millipore membrane filters membranes (0.45 and 0.20µm), and empty reagent bottles to contain the filter-sterilized solution (Figure 17). In the absence of a filtration facility, sterilize glass and millipore holders containing millipore membrane filters. Disposable sterile syringes and Millipore filters are also available.
- Filter-sterilize the GA3 stock solution using sterile 0.45µm millipore membrane filters.
- Proceed with filter-sterilization using 0.20µm millipore membrane filters.
- Compute for the required volume of GA3 needed.

For BAP-supplemented medium, co-autoclave BAP with the culture medium.

Figure 17. Filter sterilization (MBA Ubaldo, PCA-ARC)

6. Germinate the embryos in test tubes. Incubate the embryos either in the dark or 9 h light conditions (25µM/m^2/s) at 26-28°C.
7. Transfer the embryo cultures to fresh medium for the next two to three months.

Seedling elongation and further growth and development

1. Transfer seedlings to test tubes or bottles with liquid Y3 (1976) or "hybrid 2002" medium with 10mg/L BAP (cytokinins) when transferred to light. Subculture lightly-rooted seedling to Y3 medium with 37.24 g/l NAA in CRISL and 10 ppm NAA in PCAARC for 1 month. Incubate cultures under light conditions (25-45µM/m^2/s) for 9-18h light/24h at 26-28°C.
2. Depending on the laboratory technique used the haustorium and primary root may or may not be cut during sub-culturing or replenishment of medium. When using narrow-mouthed bottles i.e. ketchup bottles, it is difficult to take out the cultures, hence, haustorium and primary roots cannot be cut. However, if the lab is using wide-mouthed bottles and/or sub culturing their cultures (transferring to new medium in another container), they can easily cut the enlarged haustorium and the primary root. Normally, roots are cut when the cultures have developed shoots and the roots are not yet that profuse. Cutting the primary roots also enhances root formation.
3. Finally, subculture at one to two months intervals for four to nine months. Prepare seedlings with fully-developed shoot, at least one fully-expanded leaf and primary and secondary roots for acclimatization.

Note: In laboratories which practice medium-replenishment, culture the embryos in wide-mouthed bottles or ketchup bottles, whichever is available. With this method, the embryos are not transferred to new culture vessels during subculture; instead, the embryos and eventually, the growing seedlings are maintained in the same culture vessel. The medium is replenished with the new medium either by decanting or pumping out the old medium and pouring in the new medium after one to two months. A suction pump may be used to remove the old and add the new media.

Acclimatization and *ex vitro* establishment

1. Based on the minimum number of required palms for field planting: 90 palms for Tall and 45 palms for Dwarf coconuts and a 30% average success of the embryo culture protocol in coconut seedling production, the laboratory will need to culture at least 300 and 150 embryos for Tall and Dwarf coconuts, respectively.
2. The embryo cultured seedlings will be established in the field following the procedures presented in the "Manual on Standardized Research Techniques in Coconut Breeding" (STANTECH Manual) of COGENT. The guidelines are available on the COGENT website (http://www.cogentnetwork.org/).
3. Harden/acclimatize fully developed seedlings (with at least one fully-expanded leaf and primary and secondary roots) contained in culture vessels - i.e. large test tube, ketchup bottles or large bottles sealed with either a rubber stopper or polypropylene plastic sheets while still *in vitro* for one week inside the screenhouse. This will allow the seedlings to acclimatize with the *ex vitro* environment i.e. light intensity and temperature. Seedlings from fast-growing embryos are taken out of the laboratory for *ex vitro* establishment as early as four months after initial culture. The cultures must be treated carefully, to prevent contamination at this stage.
4. Prepare the potting media: It is recommended to fumigate the coir dust for the potting mix (coir dust: soil: sand) as in Sri Lanka or at least to sterilize the mix soil: sand (1:1) compost: sand (1:3) as in Côte d'Ivoire. It is also feasible to use non-sterile potting media coir dust: soil (1:1) as in the Philippines.
5. Plastic pots, small polybags, or clay pots can be used to plant the embryo cultured seedlings ex vitro. Clay-pots are preferred over polybags because the rigidness of clay pots does not disturb the roots. Wooden troughs have also been used, as in Côte d'Ivoire.
6. Prior to *ex vitro* planting which should be done carefully, wash out the media adhering to the seedlings. Treat the seedlings with fungicide (i.e. Benlate, Carbendazim, or Dithane) using rates and methods according to manufacturer's instructions.
7. During the first month in *ex vitro* conditions, seedlings are covered with plastic bags to maintain their relative humidity. After one week for a period of one month the plastic bag is rolled until the seedlings are exposed to natural environment.
8. Incubate the seedlings under shade for three to four months.

9. Provide seedlings with optimum cultural management such as fertilization, watering and protection from pests and diseases. Provide misting if available.
10. Transfer seedlings from small polybags and clay-pots to larger polybags as necessary and when they have established their roots.
11. Keep the seedlings in the screenhouse/nursery for 5 to 12 months until they are ready for field planting (Figure 18).

Figure 18. Embryo-cultured seedlings in pots (VB Johnson, Bioversity International).

Other considerations

National or international exchange?

Germplasm can be exchanged nationally as disinfected endosperm plugs in plastic bags / boxes packed in polystyrene boxes kept cool with ice packs whenever appropriate. Airlines do not allow packages with ice.

International germplasm exchange requires the more rigorous protocol as outlined in this guide, including the phytosanitary considerations highlighted in the section of safe movement. Considerations include:

1. Germplasm exchange in the form of embryos inoculated in cryotubes containing solid culture medium. Each tube should contain a single embryo to reduce risks of loss of material through contamination.

2. If no laboratory is available in the country of collection, then germplasm should be exchanged in the form of endosperm plugs (which are disinfected using the standard protocol) placed in plastic bags in a polystyrene box.
3. In such cases, the duration of transit should not exceed seven days; plugs should be re-disinfected with 10% commercial bleach (using standard protocol) upon arrival to reduce risks of loss of material through contamination.
4. If no laboratory is available in the receiving country, embryo-cultured seedlings (while still in culture medium and ready for acclimatization and ex vitro establishment) could be tested in germplasm exchange.

Special conditions

A humidity tent (mist system) (Orense et al., 2011) or a so called "CO_2 box" (Samosir et al., 2008) could be used for establishment of embryo cultured seedlings prior to transfer to screenhouse or nursery.

Others

Establishing a mobile laboratory for collecting could be an option.

Concluding comments

It is vital that those involved with embryo transfer ensure that:

a) The plugs (or embryos) are obtained in the best of conditions at the country of origin of the germplasm so that the plugs (or embryos) are in the best condition for transfer.
b) The plugs (or embryos) travel in the safest way to avoid damage so they to arrive in good order at receiving site to start embryo culture.
c) In order to achieve safe transfer of healthy and vigorous embryos, plan ahead in fine detail, according to the guidelines in previous sections, including aspects such as routing, number of persons travelling from the receiving site to site source of germplasm, and cork-borer design (C Oropeza 2012, pers. comm.).

However experience (e.g. see annex A), shows us that successful embryo transfer faces a number of challenges. Many scientists agree that a better option for coconut germplasm exchange could be that of establishing or strengthening the capacity for producing plantlets from excised embryos *in vitro* at the origin site (the COGENT genebanks) and then transferring these *in vitro* plantlets to other countries.

This should be done using adequate containers, but having the plantlets ready for *ex vitro* transference at reception site. Oropeza et al. (pers. comm. 2012) have conducted preliminary tests with such a container with plantlets and storage time (Figure 19). For this option there must be an investment to establish a proper laboratory for in vitro embryo germination to plantlet formation at each COGENT genebank. The plantlets produced then could probably be sent via courier to the receiving country which would then only need basic facilities to bag plantlets and keep them in a greenhouse before transferring them to a nursery. This approach still would require validation.

Figure 19. Embryo-cultured coconut plantlets for transfer (C Oropeza, CICY).

Traceability of the embryo cultured coconuts

In vitro culture of zygotic embryos is often used to introduce new accessions in national and international genebanks. Planting a new accession in a coconut genebank, or transferring an accession from one genebank to another, are quite demanding tasks. It is necessary to plant at least be 45 palms per Dwarf and 90 palms per Tall cultivar, and these new palms must have been originally generated from a sufficient number of parent palms. Each accession normally occupies 0.2 to 0.6 hectares for at least 25 years. For allogamous varieties such as Tall-type coconut palms, controlled pollination by inflorescence-bagging must be used to produce seedlings and embryos from the accessions conserved in the genebanks.

Whether the germplasm is transferred by seednuts, or by in vitro culture of zygotic embryos, or by any other means, the traceability of the genealogy of the planting material is crucial for three main reasons. First of all, traceability allows curators to know the numbers of female and male parents used at each generation and to control the genetic drift. Secondly, such traceability makes it possible to check the reliability of controlled pollination by using DNA markers. Thirdly, it allows conducting genetic studies and comparing the progenies of different parents from the same variety.

A golden rule is that, for each and every coconut palm planted in a genebank, **at least its mother palm should be known**. Knowing only the variety or population name is not enough for scientific purposes.

In fact, there are two different cases:

- **Open-pollinated seednuts collected from farmer's field.** In this case, for allogamous varieties such as Tall-type coconuts, the father palm is unknown. A unique number must be allocated to each mother palm harvested in farmers' fields. Ideally a map of farmers' fields should be drawn, using geographical system and/or satellite image. The unique number given to each harvested palm should be reported on this map. In this way, it will be possible to relocate a particular parent palm and, if needed, to return to this palm to get additional seednuts or additional characterization data. The numbers of the parent palms must then be kept and tagged to each seednut, embryo container and seedling, until after its successful establishment in the genebank. Each plot within the genebank should be mapped, and the number of the parent palm should be reported on this map, and entered in adapted computer files

in order to maintain traceability of the genealogy. For instance, curators will know that the palm located in the collection in the experimental field M63, row 12, palm on the row 23, planted in May 2009, comes from the mother palm number Z332, which is geo-referenced on the dedicated map of the farmer's field.

- **Seednuts or embryos harvested in a genebanks**. In most of the cases, for allogamous varieties such as Tall-type coconut palms, both female parents and male parents are located in research centre(s), so the seednuts should be obtained by controlled pollination. In this case, the female parent number and the male parent number must be kept carefully until after the progeny palms have been planted in the new location.

An example from the international genebank for Africa and Indian Ocean illustrates optimal traceability. In the Côte d'Ivoire genebank, the cultivar Gazelle Peninsula Tall (GPT) was first introduced in 1983 from Papua New Guinea. GPT has been recently rejuvenated for the first time. In 2007 and 2008, 157 new palms were planted in the experimental field 063. As an example, the palm located field 063, row number 7, palm number 19, and planted in 2007, comes from the pollination number AM6767. Then, by checking the catalogue of controlled pollinations, we know that its female parent is located in field 142, row 18, palm 7, and was planted in 1984. We also know that its male parent is located on plot 142, row 18, palm 02, and was planted in 1984. The curator and researchers used this information in the following ways: First, they took leaf samples from these three palms (parents and progenies). Then they extracted the DNA and conducted an analysis using eight markers from the microsatellite kit created by CIRAD for COGENT. This analysis confirmed that this particular progeny palm really originates from the two identified parents. Secondly, researchers calculated that, although 48 female and 24 male parents were initially used for making the controlled pollinations, the sample of palms presently available in the field comes from only 46 female and 24 male parents. In fact, the controlled pollinations were unsuccessful on only two female parents, so the genetic drift was reduced.

Achieving this optimal level of traceability is indeed a complex process, because **all parent palm numbers must be kept safely at all the stages**. These numbers must be written on the parent palms, on the coconuts, on the tubes containing embryos, then tagged on the seedlings in the pre-nursery, then in the nursery, and then kept in the field until after the planting. Subsequently these numbers should be recorded on a planting

map and entered in their appropriate files. Finally this information should be used to check the genealogy and calculate the numbers of female and male parent palms of each accession.

International movements of coconut embryos are used for introducing new accessions in coconut genebanks. As with any of the possible kinds of introduction of germplasm to genebanks, a standard procedure must be followed to gather and record information about the origin and characteristics of the germplasm. All the passport data and the characterization data obtained from parent palms must be recorded in a format according to the coconut standard descriptors published by Bioversity International (IPGRI 1995). Then this information will have to be inputted into the Coconut Genetic Resources Database for sharing it with curators and the whole coconut scientific community.

Movement of seednuts

(Frison and Putter, 1993)

1. Seednuts should only be transferred where circumstances prevent the extraction of embryos in the country of export or when a thorough pest risk assessment fails to show problems of quarantine concern.
2. Mature seednuts should be taken from the palm when at least one nut in the bunch turns from the fresh to the dry colour. After removing the stalks and calyces, they should be partially de-husked, leaving a layer of fibre up to 3 cm thick. Seednuts should be harvested and dispatched without delay to minimize the risk of germination before they reach the importing country.
3. In the country of export, the seednuts should be fumigated with methyl bromide at normal atmospheric pressure at a rate of 32 g/m3 for 3 h at 21°C or above, or with aluminium phosphide at the recommended dosage, and following fumigation, treated with a suitable fungicide. It should be noted that methyl bromide may affect germination.
4. After arrival in the country of destination, the seednuts should be inspected for the presence of insect pests and re-fumigated or destroyed if any are found.
5. Unless a thorough pest risk assessment has failed to show problems of quarantine concern in the country of origin, the seednuts should be sown under containment and leaf samples from

each seedling should be indexed for viroids, and CFDV where appropriate, following the procedure described below:

a. Take 2 g of leaf tissue (c. 20 cm) at the earliest opportunity from the youngest expanded leaf. Wipe the leaflets free of moisture and debris, remove mid-ribs, and place in a sealed plastic bag.
b. Keep samples cool (but do not freeze) and immediately consign by courier or air freight to the indexing laboratory, enclosing an import permit issued by the receiving country.
c. Notify the testing laboratory by telex or fax when the samples are despatched.
d. Seedlings should be released from quarantine if the results of the indexing are negative.

6. In exceptional circumstances, such as where the country of import lacks adequate post-entry quarantine facilities, seednuts should be germinated under containment in intermediate quarantine. Seedlings should be indexed for viroids and CFDV as mentioned above, and, if the results are negative, forwarded as seedlings to the importing country.

Coconut disease risks

Introduction

Whilst it is vital to ensure the health and sanitation of the coconut embryos being transferred, it is not the function of this guide to provide comprehensive information on coconut pests and diseases. The two most serious disease threats from **Lethal Yellowing Disease** and from **Cadang-cadang** are described in more detail below (from Ikin and Batugal, 2004). They typify the key etiological issue that in many cases, visual symptoms cannot provide a definitive diagnosis, and sometimes there are even no reliable diagnostic techniques, no effective means of control and no resistant/ tolerant germplasm. Some also suggest that both **Weligama wilt** and **Kerala wilt** pose serious threats, also briefly described. The next section outlines the requirements for safe movement of coconut germplasm in the context of mitigating pest and disease threats.

There are many useful information sources, particularly the 'Germplasm Health Manual for COGENT's Multi-Site International Coconut Genebank' (Ikin and Batugal, 2004). This publication also offers data sheets and pest risk analyses for a comprehensive range of pests and diseases. Arancon et al. (2011) provide another useful source of information on phytoplasmas and wilt diseases.

Lethal Yellowing Disease (LYD)

Caused by range of phytoplasma strains, which are spread by insect vectors, lethal yellowing disease (LYD) devastates coconut palms (Figure 20a) and affects many other palm species. The condition is widely reported in the Caribbean and Central America (Mexico, Belize, Guatemala and Honduras), and many parts of Africa. There are several lethal yellowing type syndromes (LYTS) caused by different phytoplasmas. (Dollet, 2009; Dollet 2012, pers. comm.). Infected plants will normally die in three to six months.

Effective control measures include: quarantine, chemotherapy[14], vector control, sanitation (containment by cutting at first detection) and the limited use of resistant varieties (in Africa).

Symptoms

The first visual symptom is premature fruit drop (Figure 20b), then blackening of new inflorescences. Necrosis is most apparent as the inflorescence emerges from the spathe. The first affected inflorescences usually show partial necrosis, subsequent inflorescences show more extensive necrosis (Figure 20c). Most male flowers are dead and no fruit is set on those affected inflorescences. Leaf yellowing (and sometimes browning) usually starts on the oldest leaves after necrosis has developed in more than two inflorescences. LY leaf senescence is quicker than normal leaf senescence. Leaf yellowing then progresses upwards, and when the yellowing reaches the youngest leaves, in most cases bud-rot and leaf-spearing occur. The rot weakens the top of the trunk and the final tuft of young stunted leaves may tilt and drop (Dollet 2012, pers. comm.) followed by necrosis, desiccation, death and leaf fall (Figure 20d). Eventually, the whole crown perishes, leaving a bare trunk or 'telephone pole' (Figure 20a). LY does not always follow the same sequence of events. In some affected palms the spear leaf or a mid-crown leaf occasionally shows premature chlorosis. Sometimes inflorescence necrosis becomes noticeable only after leaf yellowing has appeared. The latent period for LY has been variously reported from 210-450 days according to palm age and context. In addition roots also show necrosis, becoming more extensive as the disease progresses. Growth is also affected by LY. Symptoms in other palms are similar.

Diagnosis and detection

Visual symptoms allow for a tentative diagnosis but confirmation requires detection of the LY phytoplasmas in host tissues, either by direct electron microscopic observation or indirectly by DNA analysis. Diagnosis can be also supported by differential responses of diseased palms to antibiotics. DNA analysis confirms that phytoplasmas are detectable in all growing parts except mature leaves and the trunk was found to support detectable phytoplasma levels prior to symptoms appearing.

---

[14] Antibiotherapy in Florida as preventive treatments for non-food palms; curative treatments not possible; antibiotic treatments not allowed in several countries (M. Dollet 2012, Pers. comm.).

Figure 20a. LYD devastated coconut palms, Ghana (M Dollet, CIRAD).

Figure 20b. Premature fruit drop, Ghana (M Dollet, CIRAD).

Figure 20c. Inflorescence necrosis caused by LYD, Cuba (M Dollet, CIRAD).

Figure 20d. LYD leaf chlorosis, Mozambique (M Dollet, CIRAD).

Cadang cadang

Cadang cadang is caused by the Cadang cadang viroid (CCCVd), which is reportedly transmitted via the seed or more rarely by the pollen. There are several examples of other plants for which viruses occur in the seeds but they are not transmitted (they are outside the embryo cell). However there is little evidence showing that CCC was transmitted by seed or pollen (Dollet 2012, pers. comm.).

There is natural infection of all commercial coconut palm cultivars (*Cocos nucifera L.*), and of two other primary hosts: *Elaeis guineensis* (African oil palm), and Buri palm (*Corypha elata*). Some (see Ikin and Batugal, 2004) suggest that *Arenga pinnata* (sugar palm), *Areca catechu* (betelnut palm), and *Borassus* can also act as primary hosts. 'Experimental hosts' via inoculation under pressure with the purified viroid: *Adonidia merrillii, Areca catechu*, *Chloris* (fingergrasses), *Caryota cumingii, Chrysalidocarpus lutescens, Livistona rotundifolia, Phoenix dactylifera* (date palm), *Ptychosperma macarthurii Roystonea regia.*

Restricted to Oceania, mainly in the Philippines, it causes slow death over seven to 16 years, and apart from exclusion, there are no effective control measures for Cadang cadang.

Symptoms

The earliest symptoms are nut-rounding, equatorial nut-scarifications, and the appearance of fine, translucent, bright yellow leaf spots (Figure 21a, next page). Inflorescences then become necrotic, nut production declines and then ceases, frond production slows, and general chlorosis appears, followed by death of the crown (Figure 21b, next page). Artificially inoculated seedlings show varying severities of leaf spotting and stunting. Some palms die prematurely, and those that continue to develop never produce flowers. Symptoms are unreliable for diagnosis at a single observation. No resistance has been found.

Diagnosis and detection

CCCVd can be detected in coconut husks, embryos and pollen. Reliable diagnosis is achievable only by molecular methods, as disease symptoms are unreliable as a means of detecting CCCVd. Strict certification of origin is essential, and although no Seed health tests have yet been developed, CCCVd can be detected in the coconut husk and embryo by molecular DNA hybridization.

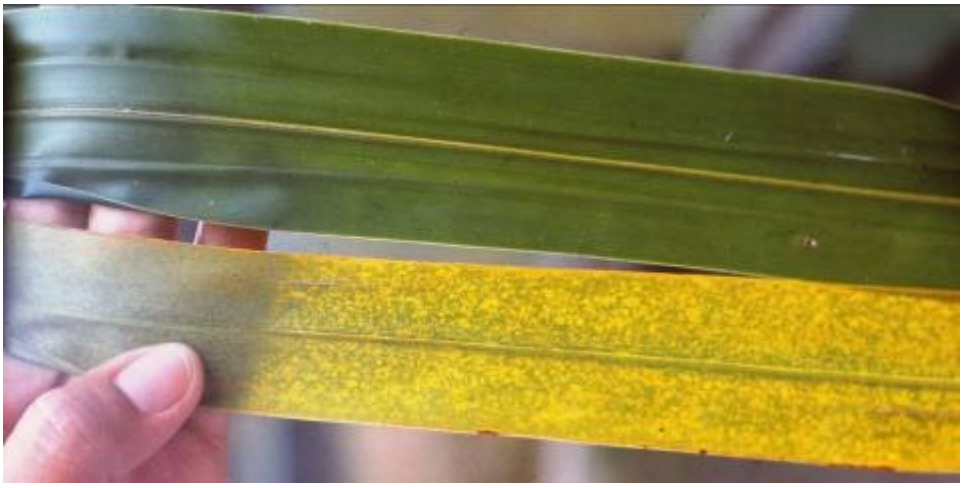


Figure 21a. Coconut leaf symptoms of Cadang cadang: healthy palm (top) and palm with late stage of the disease showing non necrotic chlorotic spotting (J Randles, Waite Agricultural Research Institute, University of Adelaide).

Figure 21b. Area with high incidence of Cadang cadang disease, showing trees in early, mid and late stages (J Randles, Waite Agricultural Research Institute, University of Adelaide).

Weligama coconut leaf wilt disease and Kerala wilt

Occurring in South Asia, mainly India and Sri Lanka, symptoms are flaccidity and marginal necrosis of leaflets and yellowing of the fronds (Figure 22). However, symptoms are virtually never distinctively diagnostic, as many other diseases and some nutrient deficiencies generate a similar range of symptoms. As the disease progresses the crown becomes smaller, the trunk begins to taper and the palm becomes unreproductive. It has been confirmed using molecular analysis that a phytoplasma belonging to the 16SrXI '*Candidatus Phytoplasma oryzae*' group is associated with Weligama coconut leaf wilt disease (Perera et al., 2012). It is also believed that the disease came to Sri Lanka either by wind transmission of phytoplasma carrying insect vectors or illegal importation of infected palms from India.

Figure 22. Coconut palm suffering reported Weligama wilt (L Perrera, CRISL).

Safe movement of coconut germplasm

Although essential to using and improving coconut genetic resources, the movement of coconut germplasm also poses a risk of transferring pests and diseases. This is particularly the case when the germplasm is moved from one major coconut-growing region to another, since some coconut pests and diseases are geographically isolated. The spread of Lethal Yellowing or Cadang cadang demonstrates why strict precautions are needed when moving coconut material. This applies both between regions and within a country. The presence of viruses in collections can impact on the genebank's ability to distribute germplasm, as can high levels of pests and diseases which can reduce the vigour of palms and thus the availability of suitable material, in addition to increasing the risk of transferring infected materials. Pest and diseases preventing distribution of germplasm have a major effect for the collections of many countries. It is essential therefore that all stakeholders handling coconut genetic resources have access to information highlighting the risks associated with each particular pest or disease and recommendations on appropriate quarantine measures.

The former section of the guidelines refers to the major pest risks associated with coconuts.

The 'Coconut germplasm health management guidelines' (Ikin and Batugal, 2004) contains detailed tables describing procedures for safe movement (e.g. pp. 36-52).

Currently there is no centralized system for the safe movement of coconut germplasm at the global level. Virus, viroid and phytoplasma indexing is available at the centres listed in the next section.

Germplasm should be obtained from the safest source possible, e.g. from a pathogen–tested collection.

The conditions and requirements that apply to most safe movement requests for germplasm should include:

a) a Standard Material Transfer Agreement (SMTA- see below) for the international collections under The International Treaty on Plant Genetic Resources for Food and Agriculture (ITPGRFA or 'the Treaty') This is the case for any material sourced from collections linked with COGENT;

b) a formal approval process from the research institutes that have the official mandate from governments requiring the establishment of official links between the requesting institutes;
c) import permits from the recipient country before sending out materials, and
d) sanitary certificates.

The full text of the Treaty can be downloaded in several languages from: http://www.planttreaty.org/content/texts-treaty-official-versions.

In summary, the Treaty aims at:

e) recognizing farmers contribution to the crop diversity that feed the world;
f)establishing a global system to provide farmers, plant breeders and scientists with access to plant genetic materials; and
g) ensuring that recipients share benefits they derive from using these genetic materials with the countries where they have been originated.

It's *Main Provisions* include:

a) A *Multilateral System*, which embraces 64 of our most important crops (including coconut) accounting for 80 percent of the food we derive from plants. The system puts these crops into an easily accessible global pool of genetic resources that is freely available to potential users in the Treaty's ratifying nations for some uses.
b) *Access and benefit sharing*: The Treaty facilitates access to the genetic materials of the 64 crops in the Multilateral System for research, breeding and training for food and agriculture. Those who access the materials must be from the Treaty's ratifying nations and they must agree to use the materials totally for research, breeding and training for food and agriculture. The Treaty prevents the recipients of genetic resources from claiming intellectual property rights over those resources in the form in which they received them, and ensures that access to genetic resources already protected by international property rights is consistent with international and national laws.
c) Those who access genetic materials through the Multilateral System agree to share any benefits from their use through four benefit-sharing mechanisms established by the Treaty.

What is a Standard Material Transfer Agreement (SMTA)?

The SMTA can be downloaded in various languages from the Treaty website at:

http://www.planttreaty.org/content/what-smta

The SMTA is a mandatory model for parties wishing to provide and receive material under the Multilateral System. It is the result of lengthy negotiation among the Contracting Parties to the Treaty and may not be varied or abbreviated in any way. However, as a template, it contains some paragraphs and sections that need to be completed to each use.

The material transfer agreements that use the standard template are private agreements between the particular providers and recipients but the Governing Body, through FAO as the Third Party Beneficiary, is recognized as having an interest in the agreements. The standard template has been developed to ensure that the provisions of the Treaty regarding the transfer of PGRFA under the Multilateral System are enforceable on users.

Germplasm transfer should take place in consultation with the relevant plant health authorities in both the importing and exporting countries. International standards for phytosanitary measures (ISPMs) as published by the Secretariat of the International Plant Protection Convention (IPPC) should be followed (see http://www.ippc.int/). The International Plant Protection Convention (IPPC) and its Regional Plant Protection Organizations work with Convention contracting parties, to develop phytosanitary measures that underpin the parties' ability to manage pest risks and the environmental, economic and social impacts of plant pests. The IPPC is governed by the Commission on Phytosanitary Measures (CPM), which meets annually to review the state of plant protection, identifies action to control the spread of pests into new areas, develops and adopts international standards and establishes procedures for the sharing of phytosanitary information. The IPPC works with Regional Plant Protection Organizations and international organizations to build phytosanitary capacity, to identify and address risks that cross national borders.

The IPPC provides an international framework for plant protection that includes developing International Standards for Phytosanitary Measures

(ISPMs) for safeguarding plant resources. ISPMs developed as of 31 July 2009 include standards for:

- procedures and references;
- pest surveillance, survey and monitoring;
- import regulations and pest risk analysis;
- compliance procedures and phytosanitary inspection methodologies;
- pest management;
- post entry quarantine;
- exotic pest emergency response, control and eradication; and
- export certification.

The IPPC also provides information exchange related to import and export requirements, pest status and regulated pest lists provided by each member country. Developing countries also receive technical assistance to support their ability to implement the Convention and the ISPMs

In accordance with IPPC regulations, any material being transferred internationally must be accompanied by a phytosanitary certificate (see IPPC website).

Box 1 (next page) provides an example of good practice by the Plant Quarantine Service of the Bureau of Plant Industry, Department of Agriculture, The Philippines.

Box 1. Good practices at BPI Plant Quarantine Service in the Philippines

In transferring plant materials the Plant Quarantine Service requires completed forms for:

1. **Exporting plant materials:** *Application for Inspection and Phytosanitary Inspection (BPI 'Q' FORM No. 10)*. Information required includes- Common Name; Scientific Name; Quantity; Description & No. of Packages; Source of Plants/Plant Products; Name & Address of Consignee; Date and Place of Inspection Desired; Port Of Entry; Means of Conveyance; Flight No./Voyage No.; Departure Date; Import Permit No./Additional Declaration/Treatment (if any); (Signature of Applicant/Authorized Representative); stamp of the service (Name & Designation of Applicant/Authorized Representative).

2. **Application for Accreditation-** as an importer of plant materials, signed by the public notary

3. **Accreditation Validation of accreditation:** Information required includes- Company Name; Address of Present Office; Contact Numbers (Tel. & Fax No.); T.I.N. Number; Name of the Owner; Name of Representative/s; Commodity to be imported.

   To be filled up by the PQ officer - Application for Accreditation (Notarized); Company Profile; DTI Registration/ SEC Registration/CDA Certificate of Registration; Current Mayor's Permit (2009); Valid Certificate/Contract of Lease of Storage Facilities; Sketch map of Storage Facilities; Special Power of Attorney of Representative/s; Current Bureau of Customs-Certification of Accreditation; Two (2) 2X2 ID Pictures of the Owner and the Representative/s; Notes:; PQ Officer signature.

4. **Application for Plant Quarantine Clearance Plants/Plant Products (BPI** 'Q' FORM No. 1). Information required includes- Common Name; Scientific Name; Quantity; Purpose of Importation; Place of Origin or Source of Plants/Plant Products; Name, Address & Country of Exporter; Port Of Entry; Means of Conveyance; Flight No./Voyage No.; Final Destination (Exact location and Sketch where plants are grown); Expected Date of Arrival; Signature of Applicant/Authorized Representative; stamp of the service (Name & Designation of Applicant/Authorized Representative).

Indexing laboratories

Waite Agricultural Research Institute
Department of Crop Protection
Glen Osmond, Adelaide
South Australia 5064
Australia

Philippine Coconut Authority-Albay Research Centre
Banao, Guinobatan, Albay,
Philippines

Coconut Research Institute
Luniluwila Estates
Sri Lanka

Laboratoire de Phytovirologie des Régions Chaudes
CIRAD
Avenue d'Agropolis
34398 Montpellier Cedex 5
France

Dr Adrian Fox
Senior Virologist, Team Leader - Detection & Surveillance,
Plant Protection Programme, FERA, Sand Hutton,
York, YO41 1LZ, UK
e-mail: adrian.fox@fera.gsi.gov.uk
Tel: +44 (0) 1904 462747
Fax: +44 (0) 1904 462111
Website: www.defra.gov.uk/fera

Contributors

Contributing institutions (by alphabetical order)

Institution	Country	Website	Logo
Bioversity International	Italy	http://www.bioversityinternational.org/	
the Central Plantation Crops Research Institute (CPCRI)	India	http://www.cpcri.gov.in/	
the Centro de Investigación Científica de Yucatán	Mexico	http://www.cicy.mx/	
the Cocoa & Coconut Institute (CCI)	PNG		
the coconut genetic resources network (COGENT)	France	http://www.cogentnetwork.org/	
the Coconut Research Institute (CRI)	Sri Lanka	http://www.cri.gov.lk/	
the Global Crop Diversity Trust	Germany	http://www.croptrust.org/	
L'Institut de recherche pour le développement (IRD) - UMR DIADE	France	http://www.diade-research.fr/	
the Ministry of Coconut Development and Janatha Estate Development	Sri Lanka	http://www.cdjedmin.gov.lk/	
the Philippine Coconut Authority	Philippines	http://www.pca.da.gov.ph/index.php	

Institution	Country	Website	Logo
Station de recherche Marc Delorme, Centre national de recherche agronomique (CNRA)	Côte d'Ivoire	http://www.cnra.ci/descprog.php?id=3&prog=Cocotier&act=present	

Individual contributors

By alphabetical order:

Roland Bourdeix
COGENT Coordinator
Honorary Research Fellow
C/o Bioversity International
Parc Scientifique Agropolis II
34397 Montpellier Cedex 5,
France
Tel.: +33 (0)4 67 61 32 85
Roland.BOURDEIX@cefe.cnrs.fr

Cristeta A. Cueto
Senior Science Research Specialist
Officer-In-charge-Division Chief
Philippine Coconut Authority
Albay Research Center
Banao, Guinobatan, Albay, Philippines
Tel.: +63 917 652 5064
cacueto@yahoo.com

Florent Engelmann
Senior Research Officer
Institut de recherche pour le développement (IRD), UMR DIADE
DIversité Adaptation et
DEveloppement des plantes
DESSITROP
911 Av. Agropolis
34394 Montpellier cedex 5, France
Tel +33 (0)4 67 41 62 24
florent.engelmann@ird.fr

Vincent Johnson
Process Manager/ Science Editor
CGRPVC programme
Bioversity International
Parc Scientifique Agropolis II
34397 Montpellier Cedex 5, France
Tel: +33 (0) 4 67 61 98 16
Mobile: +33 (0) 6 15 66 07 56
Email: v.johnson@cgiar.org
skype: vincentb.johnson

Dr Anitha Karun
Principal Scientist Horticulture
Crop Improvement Division
CPCRI
Kudlu P.O, Kasaragod,
Kerala, 671124, India
Tel: +91 4 994 232 894
Email: anithakarun2008@gmail.co
karun_ani@yahoo.co.uk

Alfred Kembu
Cocoa & Coconut Institute of PNG
P O Box 642,
Madang, Madang Province
Papua New Guinea
Tel.:+675 852 16 51 / 852 16 53
Fax: +675 852 16 57
cnbreeding@datec.net.pg ;
kembua@ymail.com
mgfaure_1608@yahoo.com.au

Jean Louis Konan
Head, Coconut Research Programme
CNRA
Station Cocotier Marc Delorme
07 BP 13 Abidjan 07,
Côte d'Ivoire
Tel. :+225 05 174 183 / 225 21 248 872
Fax: 225 23 472 411
jeanlouiskonan@yahoo.fr
konankonanjeanlouis@yahoo.fr

Modeste Kouassi Kan
Geneticist, Plant tissue culture Specialist
CNRA Laboratoire Central de
Biotechnologie (CNRA-LCB)
01 BP 1740 Abidjan 01,
Côte d'Ivoire
Office tel.: +225 23 472 414
Home tel.: +225 02 210 005 /07 869 878
Fax: +225 23472411

Carlos M. Oropeza Salín
Centro de Investigación Científica de Yucatán, A.C. (CICY)
Calle 43 No. 130,
Colonia Chuburná de Hidalgo
CP 97200, Mérida, Yucatán Mexico
Office tel.: +52 999 942 83 30
cos@cicy.mx

Ramon L. Rivera
Varietal Improvement Program Leader
Philippine Coconut Authority -
Zamboanga Research Centre
(PCA_ZRC)
San Ramon, 7000 Zamboanga City
Philippines
Tel.: +639 177 223 049 / +636 298 203 02
rlrivera_pca@yahoo.com.ph

V Vidhanaarachchi
Senior Research officer
Coconut Research Institute
Bandirippuwa Estate
Lunuwila 51132
Sri Lanka
vijitharma@yahoo.com ;
luckminiw@yahoo.com
Tel.: +94 31 2257419

Stephan Weise
Deputy Director for Research
Bioversity International
Via dei Tre Denari, 472a
00057 Maccarese (Rome), Italy
Tel: +39 066 118 225
Fax: +39 066 1979 661
s.weise@cgiar.org

References

Arancon RN, Shantichandra WKN, Priyanthie Fernando LC, Perera C, Aucith Dissanayake JD, Naynanayake A, Megahakummbura K, editors. 2011. Proceedings of the APCC/MCD&JED/CRI Consultative Meeting on Phytoplasmas/Wilt Diseases in Coconut, Coconut Research Institute, Luniwila, Sri Lanka, 15-17 June 2011.

Batugal PA, Engelmann F, editors. 1998. Coconut embryo *in vitro* culture. Proceedings of the first workshop on embryo culture, Banao, Guinobatan, Albay, Philippines, 27-31 Oct. 1997. IPGRI/APO, Serdang, Malaysia.

Bourdeix R, Johnson VB, Tuia VS, Weise S. 2012. Three declinations of the Polymotu Concept: "Inland *ex situ*", "Ecotourism on Islands", "Urban" and their possible applications in Brazil, Côte d'Ivoire, Indonesia, French Polynesia and Samoa (Paper presented at the 45th APCC COCOTECH Meeting, 2nd - 6th July 2012, Kochi, India).

De Guzman, E, del Rosario DA. 1964. The growth and development of *Cocos nucifera* L 'Makapuno' embryo *in vitro.* Philipp. Agric. 48:82-94.

Dollet M, Quaicoe R, Pilet F. 2009. Review of coconut "Lethal Yellowing" type diseases. Diversity, variability and diagnosis. Oléagineux Corps gras Lipides. (16)2: 97-101.

Eeuwens CJ. 1976. Mineral requirements for growth and callus initiation of tissue explants excised from mature coconut palms (*Cocos nucifera* L.*)* and cultured *in vitro*. Physiol. Plant. 36:23-28.

Engelmann F, Batugal P, Oliver J, editors. 2002. Coconut embryo *in vitro* culture: part II. Proceedings of second international workshop on embryo culture, Mérida, Yucatán, Mexico, 14-17 March 2000.

Engelmann F, Malaurie B, Oulo N'Nan. 2011. *In vitro* culture of coconut zygotic embryos. pp. 63-74 In: T Thorpe, E Yeung, editors. Plant Embryo Culture: Methods and Protocols, Methods in Molecular Biology Series. Humana Press, Totowa, NJ.

Frison EA, Putter CAJ, Diekman M editors. 1993. FAO/IBPGR Technical Guidelines for the Safe Movement of Coconut Germplasm. Food and Agriculture Organization of the United Nations, Rome/International Board for Plant Genetic Resources, Rome, Italy. Available from the URL: (http://www.cogentnetwork.org/index.php/technical-bulletins)

Ikin R, Batugal P, editors. 2004. Germplasm health management for COGENT's multi-site international coconut genebank. International Plant Genetic Resources Institute – Regional Office for Asia, the Pacific and Oceania (IPGRI-APO), Serdang, Selangor DE, Malaysia. Available from URL: http://www.cogentnetwork.org/index.php/manuals-and-handbooks.

IPGRI. 1995. Descriptors for Coconut (*Cocos nucifera* L.). International Plant Genetic Resources Institute, Rome, Italy.

Konan JL, Bourdeix R, George ML. 2008. Regeneration Guidelines: Coconut. In: E Dulloo E, I Thorman, MA Jorge, J Hansen, editors. Crop Specific Regeneration Guidelines (CD-ROM). CGIAR System-wide Genetic Resources Programme. Rome, Italy. 10pp.

Orense OD, Rillo EP, Imperial LAI, Cueto CA, Lobos AA, Areza-Buena MBB. 2011. Rapid and cost-effective embryo culture technique for commercial production of Makapuno seedlings. CORD 27(1):20-41.

Pech y Aké AE, Maust B, Orozco-Segovia A, Oropeza C. 2007. The effect of gibberellic acid on the *in vitro* germination of coconut zygotic embryos and their conversion into plantlets. *In Vitro* Cell.Dev.Biol.—Plant (2007) 43:247–253.

Pech y Aké, AE, Souza, R, Maust, B, JM Santamaria, JM, Oropeza.C. 2004. Enhanced aerobic respiration improves *in vitro* coconut embryo germination and culture. *In vitro* Cell. Dev. Biol.—Plant 40(1):90–94.

Perera L, Meegahakumbura MK, Wijesekara HRT, Fernando WBS, Dickinson MJ. 2012. A phytoplasma is associated with the Weligama coconut leaf wilt disease in Sri Lanka. J. Plant Pathol. 94(1):2015-209.

Rillo EP, Cueto CA, Medes WR, Areza-Ubaldo MB. 2002. Development of an improved embryo culture protocol for coconut in the Philippines. In: F. Engelmann. P. Batugal and JT Oliver, editors. Coconut embryo *in vitro* culture: part II. Proceedings of 2nd international workshop on embryo culture, Mérida, Yucatán, Mexico, 14-17 March 2000. p. 41.

Rodriguez MJBR. 2011. The nature of the Cadang-Cadang disease of coconut in the Philippines and review of the R & D Programme: Strategies and accomplishments. In: RN Arancon, editor. Final proceedings report of the APCC/MCD and JED/CRI Consultative Meeting on Phytoplasma/Wilt diseases in Coconut. Coconut Research Institute, Lunuwila, Sri Lanka.

Santos GA, Batugal PA, Othman A, Baudouin L, Labouisse JP. 1996. Manual on Standardized Research Techniques in Coconut Breeding. IPGRI, Rome, Italy.

Samosir YMS, Mashud N, Novarianto H, Vu Thi My Lien, Rillo E, Magdalita P, Damasco O, Kembu A, Faure MG, Adkins SW. 2008. A New Embryo Culture Protocol for Coconut Germplasm Conservation and Elite-type Seedling Production. In: Final Report: Development of an embryo culture manual and an embryo transplantation technique for coconut. Australian Centre for International Agricultural Research, Canberra. pp. 14-30.

Shivashankar,S, Karun A and Sajini KK 1999 Embryo Culture of Coconut, , thr CPCRI Protocol, Indian J Hort, 56 (4) 348-353 , Kasaragod, India

Annex A. Overall data summary for coconut embryo transfer

The validation phase of the project *Validation of a coconut embryo-culture protocol for the international exchange of germplasm* enabled refining and testing the applicability of the protocol for movement of coconut embryos to laboratories with varying capacities and experience in coconut embryo culture. Based on the results, varying levels of success were attained by the partner laboratories. Figures 23 to 26 present a summary of the data[15] to be found in Table A1 below.

Table A1. Overall data summary.

Element	Partner	Type	Hand-carried	Couriered
% embryo contamination	PCAZRC	WAT	43	98
		MYD	83	40
	CRISL	WAT	10	55
		MYD	15	23
	CCIPNG	WAT	78	13
		MYD	43	73
	Average	WAT	45	55
		MYD	47	45
% embryo germination (with *in vitro* manipulation)	PCAZRC	WAT	73	90
		MYD	50	83
	CRISL	WAT	54	3
		MYD	55	60
	CCIPNG	WAT	No data	No data
		MYD	No data	No data
	Average	WAT	63	46
		MYD	53	72

[15] incomplete data from PNG

Table A1 (cont'd)

Element	Partner	Type	Hand-carried	Couriered
% embryo germination (without *in vitro* manipulation)	PCAZRC	WAT	73	90
		MYD	50	83
	CRISL	WAT	3	0
		MYD	43	30
	CCIPNG	WAT	No data	No data
		MYD	No data	No data
	Average	WAT	38	45
		MYD	46	56
% coconut seedlings established for field-planting	PCAZRC	WAT	40	2.5
		MYD	15	20
	CRISL	WAT	10	0
		MYD	30	25
	CCIPNG	WAT	No data	No data
		MYD	No data	No data
	Average	WAT	25	1
		MYD	23	23

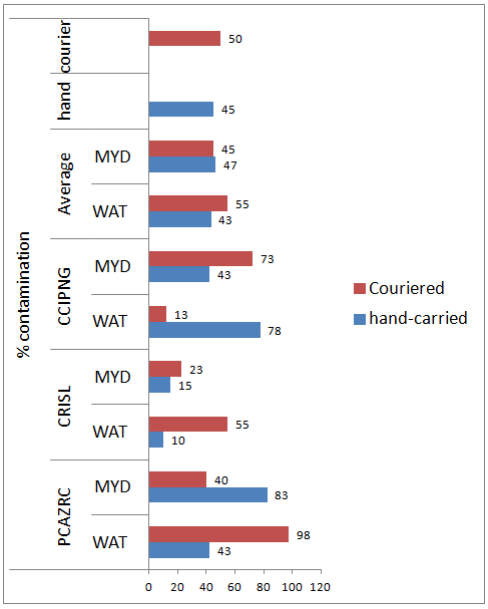


Figure 23. Coconut embryo contamination levels

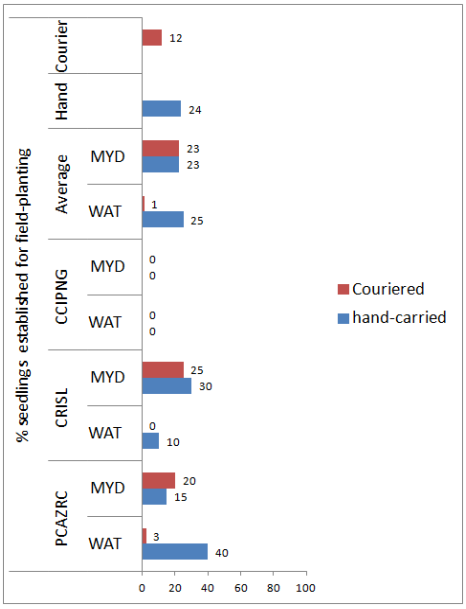


Figure 24. Coconut seedling establishment levels

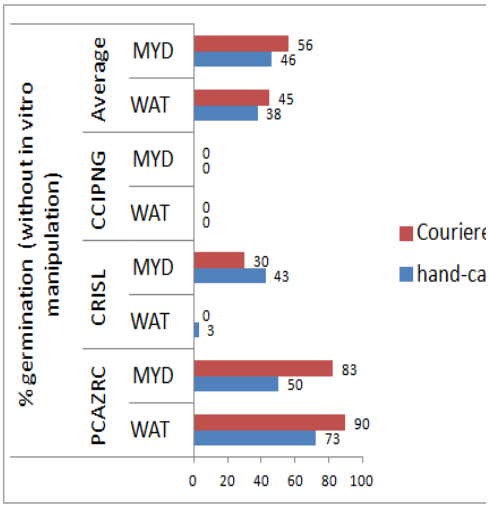


Figure 25. Germination levels of embryos without *in vitro* manipulation

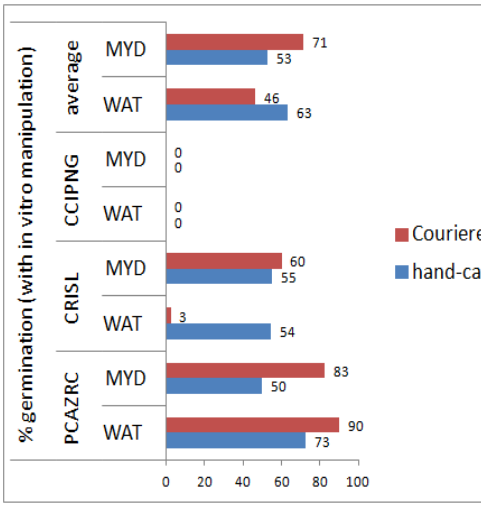


Figure 26.Germination levels of embryos with *in vitro* manipulation

Annex B. Culture medium composition

Table B1. The composition of the Eeuwen's (Y3, 1976) mineral and vitamin solutions.

COMPONENTS		Amount
MACRO		(mg/l)
Calcium chloride di-hydrate	$CaCl_2.2H_2O$	294
Magnesium sulphate heptahydrate	$MgSO_4.7H_2O$	247
Potassium nitrate	KNO_3	2020
Potassium chloride	KCl	1492
Sodium di-hydrogen phosphate di-hydrate	$NaH_2PO_4.2H_2O$	312
Ammonium chloride	NH_4Cl	535
MICRO		1x (mg/l)
Manganese sulphate tetrahydrate	$MnSO_4.4H_2O$	11.2
Zinc sulphate heptahydrate	$ZnSO_4.7H_2O$	7.2
Boric acid	H_3BO_3	3.1
Potassium iodide	KI	8.3
Cupric sulphate pentahydrate	$CuSO_4.5H_2O$	0.25
Sodium molybdate di-hydrate	$Na_2MoO_4.2H_2O$	0.24
Cobalt chloride hexahydrate	$CoCl.6H_2O$	0.24
Nickel chloride hexahydrate	$NiCl.6H_2O$	0.024
IRON		1x (mg/l)
Ferrous sulphate heptahydrate	$FeSO_4.7H_2O$	13.9
Ethylene dinitrilo tetra acetic acid disodium salt di-hydrate	$Na_2EDTA.2H_2O$	37.3

Table B1 (cont'd)

COMPONENTS		Amount
VITAMINS		1x (mg/l)
	Myo-inositol	100
Thiamine hydrochloride	Thiamine HCl	0.5
	Nicotinic acid	0.05
Pyridoxine hydrochloride	Pyridoxine HCl	0.05
Calcium pantothenate*)*	Ca D-pantothenate	0.05
	Biotin	0.05

Table B2. The components and pH of the "hybrid medium" being used for coconut embryo culture (Rillo et al., 2002).

Particulars	Source Medium	Chemicals	Weight	
			1x (mg/l)	10x (g/l)
Macro	Eeuwens (Y3)	NH_4Cl	535.00	5.35
		KNO_3	2020.00	20.20
		$MgSO_4.7H_2O$	247.00	2.47
		$CaCl_2.2H_2O$	294.00	2.94
		KCl	1492.00	14.92
		$NaH_2PO_4.2H_2O$	312.00	3.12
Micro	Eeuwens (Y3)		**1x (mg/l)**	**100x (g/l)**
		KI	8.30	0.830
		H_3BO_3	3.10	0.310
		$MnSO_4.4H_2O$	11.20	1.120
		$ZnSO_4.7H_2O$	7.20	0.720
		$CuSO_4.5H_2O$	0.250	0.0250
		$CoCl_2.6H_2O$	0.240	0.0240
		$NaMoO_4.H_2O$	0.240	0.0240
		$NiCl.6H_2O$	0.024	0.0024
EDTA	UPLB		**1x (mg/l)**	**100x (g/l)**
		$Fe_2SO_4.7H_2O$	41.70	4.170
		Na_2EDTA	55.80	5.580

Table B2 (cont'd)

Particulars	Source Medium	Chemicals	Weight	
			1x (mg/l)	10x (g/l)
Vitamins	UPLB + ARC	Pyridoxine HCl	0.05	0.005
		Thiamine HCl	0.05	0.005
		Nicotinic acid	0.05	0.005
		Ca-D-pantothenate	0.05	0.005
		Biotin	0.05	0.005
		Folic acid	0.05	0.005
		Glycine	1.00	0.1
Table grade sugar	60 g/l	• 60 g/l from culture initiation until seedlings have developed shoots and roots (until the 2nd to 3rd month)		
	45 g/l	• 45 g/l for maintenance prior to transplanting to the soil		
Activated charcoal (acid washed)		1 g/l		
Gelling agent		7-8 g/l		
State of the medium	Liquid/ solid/ liquid	• Culture initiation (So) = **liquid +60g/l sugar** • 1st Subculture (S1) = **solid + 60 g/l sugar** 2nd Subculture (S2) = **solid + 60 g/l sugar if not germinated yet** = **liquid +45 g/l sugar if germinated** • 3rd Subculture (S3) = **liquid +45 g/l sugar** until the seedlings are ready to be transplanted to the soil.		
pH		5.6		

ISBN: 978-92-9043-924-0